A New Assay for Rhamnolipid Detection – Important Virulence Factors of *Pseudomonas aeruginosa*

Maisem Laabei, [1, 2] William D. Jamieson, [1] Stephen P. Diggle, [3] A. Tobias A. Jenkins[1, a]

[1] Department of Chemistry, [2] Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, UK. [3] School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

[a] Corresponding author
Tel: 01225386118
Email: a.t.a.jenkins@bath.ac.uk

Abstract

Rhamnolipids [RL] are heterogeneous glycolipid molecules that are composed of one or two L-rhamnose sugars and one or two β-hydroxy fatty acids, which can vary in their length and branch size. They are biosurfactants, predominantly produced by *Pseudomonas aeruginosa* and are important virulence factors, playing a major role in *P. aeruginosa* pathogenesis. Therefore, a fast, accurate and high-throughput method of detecting such molecules is of real importance. Here, we illustrate the ability to detect RL producing *P. aeruginosa* strains with high sensitivity, based on an assay involving phospholipid vesicles encapsulated with a fluorescent dye. This vesicle-lysis assay is confirmed to be solely sensitive to RL. We illustrate a half maximum concentration for vesicle lysis (EC_{50}) of 40 μM (23.2 μg/mL) using pure commercial RL, and highlight the ability to semi-quantify RL directly from the culture supernatant, requiring no extra extraction or processing steps or technical expertise. We show that this method is consistent with results from thin layer chromatography detection and dry weight analysis of RL, but find that the widely used orcinol colorimetric test significantly underestimated RL quantity. Finally, we apply this methodology to compare RL production among strains isolated from either chronic or acute infections. We confirm a positive association between RL production and acute infection isolates ($p = 0.0008$), highlighting the role of RL in certain infections.

Keywords: Rhamnolipids, *Pseudomonas aeruginosa*, Lipid vesicles, Detection

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen, ubiquitous in the environment and capable of causing a multitude of infections in the immune-compromised host (Bodey et al. 1983; Driscoll et al. 2007). The ability to cause such a wide array of infections is dependent on the expression of virulence factors (Strateva and Mitov 2011). Virulence factor regulation in *P. aeruginosa* is achieved through a density-dependent cell-to-cell communication network, involving three main quorum sensing systems; the *las*, *rhl* (Schuster et al. 2013; Smith and Iglewski 2003) and Pseudomonas quinolone signal system (Dubern and Diggle 2008; Pesci et al. 1999). The *las* and *rhl* systems are LuxRI homologues, where *lasI* and *rhlI* direct synthesis of *N*-3-oxododecanoylhomoserine lactone (3-oxo-C12-HSL) and *N*-butanoylhomoserine lactone (C4-HSL) respectively; these are diffusible signalling molecules which activate their respective DNA binding response regulators, LasR and RhlR, which in turn induces the expression of a wide range of genes, approximately 6% of the genome (Schuster and Greenberg 2006; Schuster et al. 2013; Wagner et al. 2004; Williams et al. 2007). Another cell-to-cell signalling system responds to the quinolone compound 2-heptyl-3-hydroxy-quinolone (the Pseudomonas quinolone signal, PQS), acting with the transcriptional activator, PqsR (Diggle et al. 2006; Pesci et al. 1999). An elegant hierarchy system predominates in this global regulatory network, with the *las* system positively regulating both the *rhl* and quinolone signalling systems (Pesci et al. 1999; Pesci et al. 1997; Williams and Camara 2009).

One of the most interesting extracellular factors produced by *P. aeruginosa* are rhamnolipids [RL]. RL are surfactant-acting molecules, composed of a hydrophilic head of one or two rhamnose molecules and a hydrophobic tail portion of one or two fatty acids. The amphiphilic nature of RL allows these biosurfactans to partition into biological and artificial membranes altering their biophysical properties, previously shown in model membranes using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles and purified RL (Aranda et al. 2007; Sanchez et al. 2010). RL are also very important virulence factors for *P. aeruginosa*, central in immune cell and erythrocyte destruction (Fujita et al. 1988; Jensen et al. 2007), swarming and twitching motility (Caiazza et al. 2005; Deziel et al. 2003), biofilm formation and protection (Alhede et al. 2009; Pamp and Tolker-Nielsen 2007). Furthermore, they have been implicated in the deterioration of patients with ventilator associated pneumonia (Kohler et al. 2010) and disruption and permeablization of epithelial cells, a prerequisite to *P. aeruginosa* invasion (Zulianello et al. 2006).

Clearly, an accurate and rapid method to assess RL production is important. Current methods can be qualitative; the cetyltrimethylammonium bromide (CTAB) methylene blue (MB) agar test (Pinzon and Ju 2009), signals RL expression through the formation of blue halos due to the complexation of the anionic RL and cationic CTAB and MB. Although this method is quick, it suffers from a time delay due to incubation for 48 hr for optimal results and also through distortion of halo formation due to fluorescent pigments produced naturally by certain *P. aeruginosa* strains. Other methods measure the tensioactive properties of the surfactant (Morikawa et al. 2000) however these methods employ sensitive instruments and are laborious, not amenable to high-throughput. Quantitative methods consist of spectrophotometric analysis, using the orcinol test (Koch et al. 1991)), chromatographic methods including thin layer chromatography (TLC) high performance liquid chromatography (HPLC) (Schenk et al. 1995), infrared spectroscopy (IR) (Leitermann et al. 2008) and dry weight analysis of rhamnolipids following solvent extraction (Gunther et al. 2005). Here we present a new methodology in detecting and quantifying RL using carboxylfluorescein encapsulated phospholipid vesicles. We have previously shown that these vesicles are stable under various conditions (Marshall et al. 2013) and are susceptible to a suite of *Stapylococcus aureus* toxins that have surfactant-like properties (Laabei et al. 2014b). In this report we confirm that RL can be detected and semi-quantified directly from pure overnight culture supernatants of *P. aeruginosa* using the vesicle-lysis assay, decreasing preparation time, hazardous extraction techniques or expert analysis.

Materials and methods

Bacterial strains and growth conditions

P. aeruginosa strains that were used in this study were routinely stored at -80°C in 15% glycerol/broth stocks until required. These strains were maintained in Luria-Bertani (LB) medium (Fluka). A single colony was inoculated in 5 mL of LB broth contained within a 25 mL glass universal reaction tube for 20 h at 37 °C and shaking at 180 rpm. These cultures were subsequently sub-cultured 1:1000 in LB broth and grown for 18 h to generate supernatants used in this study. Bacterial growth and vesicle fluorescence was measured using absorbance OD_{600} and excitation and emission wavelengths of 485-520nm respectively. Bacterial cultures and vesicle were co-incubated at 37°C with shaking at 300 rpm in a 96-well round-bottom plate using a FLUOROstar fluorimeter (BMG LABTECH). Antibiotics were added to the media at the following concentrations for maintaining particular mutant

strains: PAO1Δ*rhlI* (tetracycline 200 µg/mL) and strains PAO1Δ*lasR* and PAO1Δ*lasI* (gentamicin 100 µg/mL).

Lipid vesicle development

Vesicles were developed as described previously (Laabei et al. 2012). Briefly, vesicle suspensions were prepared using the molar ratio of 25% 10, 12-tricosadiynoic acid (Sigma), 53% 1,-2 dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 2% 1, 2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine (DPPE) and 20% cholesterol. Lipid films were rehydrated in 50mM 5(6)-carboxyfluorescein (Sigma) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (pH 7.4), freeze-thawed three times using liquid nitrogen and extruded through a 100 nm membrane (Liposofast). Vesicles were further purified through a NAP-25 column (GE Healthcare) kept at 4°C overnight and cross-linked using a UV cross-linker (UVP CL1000). Size distribution and concentration of vesicle was measured via dynamic light scattering (Malvern) and nanosight tracking analysis (Nanosight Ltd) respectively, highlighting a vesicle size distribution of 90-110 nm and concentration of $1x10^{8}$ vesicles / µL.

Vesicle –lysis assay

The parameter for measuring the fluorescence intensity of lysed vesicles was set at excitation and emission wavelengths of 485-520nm respectively and a gain of 550 using a FLUOROstar fluorimeter (BMG LABTECH). Two types of experiments were designed, one including whole bacterial cells and one with harvested supernatant. In the former method, bacterial starting inoculums were normalised to specific concentrations of 1×10^{6}, 1×10^{5} and 1×10^{4} CFU/mL. The bacterial culture (200 µL) was added to the vesicle solution (50 µL) in triplicate and the fluorescence intensity was measured at 5 min intervals for 18 h. For the supernatant-vesicle assay, bacterial supernatants were harvested from 1 mL of 18 h culture after centrifugation for 10 min at 14,000x*g* using a bench top centrifuge. Bacterial supernatant (50 µL) and vesicle solution (50 µL) were incubated for 30 min using the same parameters as above. Normalised fluorescence was achieved using the equation (Ft-F0) / (Fm/F0) where Ft is the average fluorescence value at a specific time point, F0 is the minimum and Fm is the maximum fluorescence value in that particular experiment, determined using the negative and positive controls of HEPES buffer and 0.1% Triton X-100 respectively. In order to understand which exoprotein(s) were causing vesicle lysis, PAO1

bacterial supernatant was heated to 95°C for 1 hour and its lytic potential assessed using the above parameters. All experiments were done in triplicate three times and the error represents the 95 % confidence interval.

Thin-layer chromatography

Rhamnolipids were extracted from filtered culture supernatants using ethyl acetate in a 1:1 (v/v) ratio. Samples were mixed by vortexing with subsequent phase separation by centrifuging for 1 min at 14,000g. The upper, rhamnolipid containing phase was transferred to a new Eppendorf tube and the procedure repeated three times. The organic solvent was removed by evaporation using a vacuum centrifuge. For detection of rhamnolipids, this dried pellet was dissolved in 10 µL of ethanol. This solution (5 µL) was spotted on silica 60 TLC – plates (Fisher). In addition, 0.1% rhamnolipid solution (5 µL) containing mono- and di-rhamnolipid (R-95 Sigma) was used as a standard. TLC was performed using chloroform/methanol/acetic acid in a ratio of 65:15:2 as a developing solvent. For visualisation, the dried plate was briefly submerged in a detection agent composed of 0.15 g orcinol, 8.2 mL sulphuric acid (60%, v/v) and 42 mL deionised H_2O. The plate was left to dry at room temperature and then the sugar moieties were stained by incubating the plates at 110°C for 10 min.

Orcinol assay

Culture supernatants were obtained by centrifugation and 300 µL of this was extracted twice with 1 mL of diethyl ether. The samples were pooled and evaporated to dryness using a vacuum centrifuge and then 0.5 mL of sterile H_2O was added. To each 100 µL sample, 900 µL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. This solution was heated to 80°C for 30 min, after which the samples were cooled at room temperature for 15 min. The absorbance of the samples were measured at 421nm (BMG LABTECH) and concentration of rhamnolipids compared to those generated using a standard.

Dry Weight Analysis

Dry weight analysis was based on the procedure by *Gunther et al* (Gunther et al. 2005). Briefly, supernatant was separated from overnight culture (100 mL) by centrifugation at 7,000x*g*. Supernatant was then acidified to pH 2.0 by the drop-wise addition of 12 M hydrochloric acid. This solution was then centrifuged at 13,000x*g* and the RL containing precipitate was extracted three times with a chloroform-ethanol (2:1) mixture. This was then

evaporated away leaving the characteristic honey-like appearance. This oily residue was dissolved in methanol and transferred to a previously weighted container, where the methanol was evaporated giving the total rhamnolipid yield.

Results

Bacterial mediated lysis of vesicles occurs during early stationary phase of growth

In order to investigate at what stage of growth *P. aeruginosa* PAO1 wild type lysed lipid vesicles, different starting inoculums were used to initiate growth in a mix of nutrient rich broth and lipid vesicles (Fig. 1(a)). Expression of specific genes is essential at different stages of growth, particularly in the expression of virulence factors (Winzer and Williams 2001), capable of lysing lipid vesicles. Therefore, we investigated the ability of *P. aeruginosa* to break down vesicles and correlated this with the respective growth rate with and without vesicles within the media (Fig. 1(b)). Differences in optical density (OD_{600}) observed between the two experiments are most probably due to the vesicles impacting on light scattering, resulting in higher absorbance values. Nonetheless, vesicles did not have a negative impact on bacterial growth (Fig. 1(b)). By using different starting inocula of $1x10^4$-$1x10^6$ CFU/mL we illustrate that a time delay exists in lysis of vesicles with respect to smaller starting inoculum. With a starting inoculum of $1x10^6$ CFU/mL bacteria reach late exponential/early stationary phase of growth after approximately 400 min (Fig 1b) (blue line in both Fig 1 a and b represent 400 min time point). With a starting inoculum of $1x10^6$ CFU/mL this is the approximate time point when vesicles begin to breakdown followed by increasing fluorescence release over the next 150 min (Fig. 1(a)). This signifies that bacterial mediated breakdown of vesicles occurs during early stationary phase, when the local concentrations of bacteria reach a critical threshold, leading to the expression of quorum sensing (QS) regulatory genes known to be involved in the expression of virulence factors capable of causing membrane damage.

Identification of rhamnolipids as the vesicle lysing agent

The production of toxins/enzymes in *P. aeruginosa* is governed by a hierarchical cell to cell QS system, which when activated, leads to the production of a whole suite of virulence factors, some of which are excreted into the extracellular environment. Therefore, we investigated the ability of QS mutants to lyse lipid vesicles (Fig. 2(a)), in order to determine factor(s) involved.

The quinolone signalling system contains the *pqsABCDE* operon, in which the *pqsABCD* section is required for the synthesis of the hydrophobic quinolone signal which interacts with a LysR-like regulator PqsR, modulating the expression of genes known to function in virulence (Diggle et al. 2007; McKnight et al. 2000). Therefore, we assayed the culture supernatant of a *pqsA* mutant to identifying any deleterious effects on lipid vesicle lysis, however no difference between WT and mutant strain was evident (Fig. 2(a)). It has been shown previously that a *pqsE* mutant, although not impacting on the production of the PQS signal, negatively affects PQS-controlled virulence factors (Diggle et al. 2003; Gallagher et al. 2002) and has been shown to enhance the *rhl* system (Farrow et al. 2008). However, no difference was seen in lysis of lipid vesicles with culture supernatants derived from the *pqsE* mutant (Fig. 2(a)). Unlike the signalling molecules of the *las* and to an extent, *rhl* system, the PQS is very hydrophobic, inhibiting free diffusion between bacterial communities (Mashburn-Warren et al. 2009). To circumvent this, these PQS signals are encapsulated within membrane vesicles (MVs), derived from the outer membrane of the bacterial cell envelope (Mashburn and Whiteley 2005). Interestingly, PQS has been shown to induce membrane curvature in erythrocytes, leading to haemolysis (Schertzer and Whiteley 2012). The formation of membrane vesicles requires the expression of PqsH, which is a monooxgenase, essential for the conversion of 2-heptyl-4-quinolone (HHQ) to PQS (Gallagher et al. 2002). Therefore, in a *pqsH* mutant there is a reduced capacity to form membrane vesicles due to reduced quinolone formation to induce membrane blebbing and structural perturbations. However, no difference was seen in vesicle lysis with culture supernatants derived from this mutant and WT, suggesting that the quinolone signalling system did not play a role in lysis of this specific vesicle type (Fig. 2(a)).

The *lasRI* and *rhlRI* regulatory systems are the most well characterised QS systems in *P. aeruginosa*. Therefore, in order to investigate what secreted factor caused lysis we examined culture supernatants from deletion mutants of the signal synthase (*lasI* and *rhlI*) and the response regulators (*lasR* and *rhlR*) (Fig. 2 (a)). The results demonstrated that an active *rhlRI* system was required for vesicle lysis. *P. aeruginosa* expresses three quorum-regulated phospholipases C (PLC) enzymes: a haemolytic PLC (PclH), a non-haemolytic PLC (PlcN) (Ostroff et al. 1990) and a PLC involved in phospholipid chemotaxis (Barker et al. 2004) (PlcB). PlcH causes cytolysis, with a preferred substrate affinity for phosphotidylcholine (PC) and sphingomyelin (Ostroff et al. 1990), importantly PC is the most abundant lipid in our vesicle. For this reason, we examined the effect of a PlcH deletion

mutant on the capacity to lysis the vesicle (Fig. 2(b)). By using serial dilutions of supernatants, it was evident that PlcH did not have any effect on lysis. Certain proteins are inactivated at high temperatures, therefore to understand what was causing vesicle breakdown, supernatants were heat treated and compared to non -treated supernatants (Fig. 2(b)), however no difference in vesicle lysis ability was observed. This suggested that a heat-resistant glycolipid could be involved in overall vesicle lysis.

RL are biosurfactant glycolipids, in which the synthesis of these molecules is under the control of the *rhlABC* operon, where the *rhlA* gene is itself directly regulated by the *rhl* QS system (Pearson et al. 1997). We performed TLC on solvent extracts in order to detect RL from *P. aeruginosa* strains that were either vesicle lysis positive or negative (Fig. 2(c) and Table 1), resulting in those strains rupturing vesicles being RL positive and those being lysis negative having no detectable RL present. In order to understand conclusively what caused *P. aeruginosa* mediated breakdown of lipid vesicles, culture supernatants from an *rhlA* mutant were used and no vesicle lysis was observed (Fig. 2(d)). This *rhlA* gene is critical in the formation of RL precurors (Deziel et al. 2003) and thus without this neither mono- nor di- RL are formed.

Estimation of secreted rhamnolipid concentration from P. aeruginosa

To investigate whether this vesicle-lysis assay could be used to quantify RL secreted from culture supernatants, we created a standard curve (Fig. 3(a)) incubating vesicles and purified R-95 rhamnolipid, a mixture of the highly abundant mono- and di-rhamnolipids congeners from *P. aeruginosa*. This graph illustrates the EC_{50}, the concentration of purified rhamnolipid to cause 50% lysis of vesicles, as 23.2 μg/mL (40μM). Using this value, the quantity of RL from culture supernatants can be estimated (Fig. 3(b)), by graphing the respective normalised fluorescence obtained from serial dilutions of culture supernatants, using a sigmoidal curve fit, generating the best fit line, giving the dilution constant required for EC_{50} and multiplying to give an estimation of RL in the original culture (92.8 μg/mL for PAO1). This PAO1 RL value generated from the vesicle-lysis assay was relatively consistent with that of our dry weight analysis of PAO1 RL (172.5 ±56.4 μg/mL). It is important to note that quantity and structure of RL is dependent on many factors, including carbon source, reaction vessel and conditions, temperature and strain-specific details and that very high concentration of RL have been recorded using optimised growth medium and bioreactors (Perfumo et al. 2013;

Rikalovic et al. 2012). The growth conditions and reaction conditions we have employed are not conducive to high levels of RL production, as this was not the aim of this study.

Comparison of vesicle assay with orcinol and TLC assays

A comparison was made between the vesicle-lysis assay and the orcinol and TLC methods of RL detection and quantification. We selected 16 strains, 8 derived from acute strains and 8 derived from chronic strains and measured their respective RL content using the above methods (Table 1). The orcinol assay was chosen as this colorimetric assay is widely used. However, this method provided a significant underestimation of RL values compared to our results, perhaps due to loss of RL during the extraction procedure, which were produced at small quantities initially. This was not the first study which has highlighted erroneous results using the orcinol assay (Perfumo et al. 2013). The orcinol method can also suffer from contamination from the growth media and also other components of the cellular envelope which has rhamnose as a component in their structure, namely lipopolysaccharides. The TLC method was used qualitatively and complemented the results of our vesicle-lysis assay, where vesicle lysis positive strains were also shown to secrete RL (Table 1). The CTAB method can suffer from distortion of RL complexation circles due to pigment production (Pinzon and Ju 2009) and we were not able to generate consistent results using this method. The RL values of clinical isolates examined here (Table 1), are consistent with values shown by other groups (Rikalovic et al. 2012). Although the vesicle-lysis assay is dependent on fluorescence detection from lysed vesicles, this is not influenced by fluorescent molecules being expressed by *P. aeruginosa* (data not shown): pyoverdine is typically excited by low wavelength light (UV) whilst carboxyfluorescein is excited by blue (490 nm) light (Meyer and Abdallah 1978).

Rhamnolipid expression is associated with acute infections

It has been shown previously that RL are important virulence factors as they have been implicated in cell death and essential in correct biofilm construction and protection. We wanted to explore the use of this assay to determine rhamnolipid expression among clinical isolates. We performed vesicle-supernatant experiments on a range of clinical strains (n=78), from a chronic (n=48) and an acute (n=30) infection background to gain an understanding of the clinical importance of RL in these two classes of infection (Fig. 4). There was a positive association between RL expression and acute infection isolates (Fisher test $p = 0.0008$), which suggests that RL play an active role during acute infections.

Discussion

The vesicle-lysis assay is responsive to quorum sensing regulated factors, which are expressed during the transition from late exponential to early stationary phase of growth (Fig. 1). *P. aeruginosa* expresses a wide spectrum of exofactors, some of which are important in membrane damage. Using isogenic mutants in key regulatory QS genes and virulence determinants we investigated which factor(s) were important in the observed lysis. Due to their hydrophobic nature, PQS molecules induce the formation of MVs through an interaction with lipopolysaccharide on the bacterial outer membrane facilitating cell-to-cell communication, dependent on the expression of the *pqs* operon (Mashburn and Whiteley 2005). Interestingly , it has also been reported that exogenously added PQS molecules can also induce membrane curvature in erythrocytes, lacking any of the receptors important for MV formation, leading to haemolysis in a concentration dependent manner (Schertzer and Whiteley 2012). Additionally, after the blebbing of MVs from the bacterial cell envelope these structures are then able to fuse with recipient cells, transferring their cargo in an elegant transport mechanism (Schertzer and Whiteley 2012). Considering these observations we investigated whether the PQS signalling system was influencing lysis of vesicles used in this study. Genetic inactivation of *pqsA*, *pqsE* or *pqsH* conferred no reduction in vesicle lysis (Fig. 2(a)), suggesting no role for the PQS system in disruption of these vesicles.

Surprisingly, the phospholipase C enzyme (PlcH), a key component in the degradation of lipids due to their affinity for phosphatidylcholine head groups, did not play a role in lipid vesicle lysis as no reduction in fluorescence was observed in the *plcH* mutant or heat treated supernatants (Fig. 2(b)). Work on phospholipase 2 (PLA) has shown that the degree of saturation and most importantly, the acyl chain length of the phospholipids impact on the ability of this lipase to lyse lipid bilayers (Haimi et al. 2010). The structure of the vesicles in this study are composed of DPPC and DPPE, which are saturated phospholipids containing 16 carbon chain length fatty acids which may not be efficient substrates for PLC activity.

Inactivation of the *rhl* QS system leads to a reduction in vesicle lysis (Fig. 2(a)). RL expression is under the control of *rhlABC* operon, where *rhlA*, a gene which encodes a rhamnosyltransferase which catalyses the transfer of L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkonic acid (HAA) and is required for subsequent RL formation, is regulated by the *rhl* system. RL are also heat-resistant molecules, and our results show heat-treatment was insufficient to inhibit the lytic function of bacterial supernatants derived from

P. aeruginosa strain PAO1 (Fig. 2(b). Finally, deletion of the *rhlA* gene confers a non-lytic phenotype, consistent with TLC results showing no RL production in strains which do not lyse vesicles (Fig. 2(c-d)), confirming RL as the sole vesicle lytic agent.

As a result of these experiments, we sought to develop a new method to detect RL based on the lysis of carboxyfluorescein-encapsulated phospholipid vesicles. Here we illustrate that this assay is more rapid, sensitive and easier to perform than current methods. The vesicle-lysis assay requires no extraction procedure and therefore is not susceptible to contamination or sample loss. We have shown that this assay is semi-quantitative and can estimate the amount of RL present in culture supernatants (Fig. 3). We observe that our results are in contrast to the values generated using the orcinol assay but are consistent with TLC and dry weight analysis. However, the orcinol test relies on the extraction of RL from the supernatant, the measurement of the rhamnose content by use of orcinol and concentrated sulphuric acid and applying these absorbance values to a standard curve (Koch et al. 1991). This procedure can be susceptible to contamination from other rhamnose containing molecules such as lipopolysaccharides or from un-extracted media components and can also give erroneous results due to differences in incubation temperature and sample loss (Perfumo et al. 2013).

RL production also had a positive association with isolates derived from acute infections. It is known that genetic changes occur during chronic infections, which can lead to the down regulation of extracellular virulence factors (Van Delden and Iglewski 1998) with loss of function mutations in the central regulator *lasR* being most frequent in chronic infections, but other QS mutants involving the *rhl* system are evident (Hoffman et al. 2009; Smith et al. 2006; Wilder et al. 2009). Since *las* and *rhl* system are intricately linked, with the *las* system controlling the *rhl* system at a transcriptional and post-translational level, it was conceivable that mutations in *lasR* were causing this RL negative-phenotype in certain clinical strains. However , in PAO1 we did not see any statistically significant difference in lysis potential between *lasR* mutant and WT strains. This leads us to believe that mutations in either the *rhl* system or the *rhlABC* operon are the most likely reasons for RL negative phenotype. Novel point mutations in quorum regulators genes and mutations in the multitude of quorum regulators may also be responsible for the RL negative phenotype and work into elucidating this in ongoing.

Following form these observations, we envisage that this vesicle lysis test may be applied as a rapid phenotypic assay, useful in screening large numbers of clinical strains in an effort to determine novel mutations that may affect the expression of this important virulence factor. Given the specificity of this assay it could be an important tool in analysing the highly complex and interconnected quorum sensing systems of *P. aeruginosa*. The effects of mutation on candidate putative regulatory genes or single base pair mutations of known regulators could be assayed quickly to in order to determine their effect on a key QS-regulated virulence factors.

Determining whether an isolate is a strong- or weak-RL producer maybe important considering that specific concentration of RL are required to lyse important immune cells and disrupt the cellular barrier which prevent invasion and dissemination (Jensen et al. 2007; Zulianello et al. 2006). We anticipate that this vesicle-lysis assay may be used to semi-quantify RL production from a set of clinical strains isolated from different infections in order to assess whether RL is an important virulence factors for that specific infection, with our preliminary results suggesting that RL production is more important in acute rather than chronic infections. One limitation of this assay is that it is susceptible to other microbial surfactants, notably the phenol-soluble modulin (PSM) peptide toxins of *S. aureus* (Laabei et al. 2014a). Therefore, isolation of pure *P. aeruginosa* isolates is required before this assay can be used to detect and semi-quantify RL production.

Acknowledgment

We would like to thank Prof. Mark C. Enright (University of Bath) and Southmead Hospital (Bristol, UK) for clinical bacterial strains, Dr. Stephan Heeb (University of Nottingham, UK) for PAO1 quorum and *rhlA* mutant strains and Dr Mathew J. Wargo (University of Vermont, USA) for PAO1Δ*plcH* mutant strain. We also acknowledge the European Commission's 7th Framework programme for funding via the EC-FP7 consortium project no.245500 *Bacteriosafe* and The Royal Society and NERC grant ref: NE/J007064/1.

The authors declare they have no conflict of interest.

Table and figure captions

Table 1. Detection and quantification of rhamnolipids

Fig. 1 Lysis of vesicles occurs during early stationary phase of growth of *Pseudomonas aeruginosa*. A) The breakdown of vesicles by different starting inoculums (10^6-10^4 CFU/mL) was measured over 18 h of growth, shown here by the detection of carboxyfluorescein released from lysed vesicles. **B)** The growth curves of *P. aeruginosa*, with and without vesicles added to the medium, are illustrated over 18 hours from a starting inoculum of 10^6 CFU/mL.

Fig. 2 Identification of vesicle lysing agent. **A)** Culture supernatants of several quorum-sensing mutants (Δ*pqsA*, Δ*pqsE*, Δ*pqsH*, Δ*lasR*, Δ*lasI*, Δ*rhlR*, Δ*rhlI*) were tested against lipid vesicles, highlighting the important role of the *rhlRI* QS system in vesicle lysis. **B)** Culture supernatants of wild-type (WT) PAO1, a lipase mutant PAO1Δ*plcH* and heat treated (HT) PAO1 culture supernatant at various dilutions (neat, 75, 50, 25, 10, 1) were tested against lipid vesicles, with no significant differences observed. **C)** TLC was used to detect the presence of rhamnolipid from two strains, PAO1 (vesicle lysis positive) and PA45100 (vesicle lysis negative) **D)** Comparison of WT PAO1 and the isogenic *rhlA* mutant .

Fig. 3 Estimation of rhamnolipids in culture supernatants. A) Vesicle – purified rhamnolipid interaction illustrating the EC_{50}, the amount of purified rhamnolipid required to cause 50% of vesicle lysis as measured by fluorescence release. **B)** Estimation of rhamnolipid concentration via serial dilutions of PAO1 culture supernatant exposed to vesicles, generating different fluorescence values. EC_{50} value obtained using 25% supernatant, thus EC_{50} times 4 giving the estimated starting RL quantity (23.2 μg /ml x 4 = 92.8 μg /ml)

Fig. 4 Positive association between rhamnolipid expression and isolates from acute infections. Vesicle – supernatant assay exploring the rhamnolipid producing capacity of clinical *P. aeruginosa* isolates derived from either a **A)** chronic (n=48) or **B)** acute infection (n=30). A positive association between rhamnolipid production and isolates from acute infections was observed, using an exact fisher test, p=0.0008.

References

Alhede M, Bjarnsholt T, Jensen PO, Phipps RK, Moser C, Christophersen L, Christensen LD, van Gennip M, Parsek M, Hoiby N, Rasmussen TB, Givskov M (2009) Pseudomonas aeruginosa recognizes and responds aggressively to the presence of polymorphonuclear leukocytes. Microbiology 155(Pt 11):3500-8 doi:10.1099/mic.0.031443-0

Aranda FJ, Espuny MJ, Marques A, Teruel JA, Manresa A, Ortiz A (2007) Thermodynamics of the interaction of a dirhamnolipid biosurfactant secreted by Pseudomonas aeruginosa with phospholipid membranes. Langmuir 23(5):2700-5 doi:10.1021/la061464z

Barker AP, Vasil AI, Filloux A, Ball G, Wilderman PJ, Vasil ML (2004) A novel extracellular phospholipase C of Pseudomonas aeruginosa is required for phospholipid chemotaxis. Mol Microbiol 53(4):1089-98 doi:10.1111/j.1365-2958.2004.04189.x

Bodey GP, Bolivar R, Fainstein V, Jadeja L (1983) Infections caused by Pseudomonas aeruginosa. Reviews of infectious diseases 5(2):279-313

Caiazza NC, Shanks RM, O'Toole GA (2005) Rhamnolipids modulate swarming motility patterns of Pseudomonas aeruginosa. J Bacteriol 187(21):7351-61 doi:10.1128/JB.187.21.7351-7361.2005

Deziel E, Lepine F, Milot S, Villemur R (2003) rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology 149(Pt 8):2005-13

Diggle SP, Cornelis P, Williams P, Camara M (2006) 4-quinolone signalling in Pseudomonas aeruginosa: old molecules, new perspectives. International journal of medical microbiology : IJMM 296(2-3):83-91 doi:10.1016/j.ijmm.2006.01.038

Diggle SP, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, Kong X, Hider RC, Cornelis P, Camara M, Williams P (2007) The Pseudomonas aeruginosa 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chem Biol 14(1):87-96 doi:10.1016/j.chembiol.2006.11.014

Diggle SP, Winzer K, Chhabra SR, Worrall KE, Camara M, Williams P (2003) The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Mol Microbiol 50(1):29-43

Driscoll JA, Brody SL, Kollef MH (2007) The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections. Drugs 67(3):351-68

Dubern JF, Diggle SP (2008) Quorum sensing by 2-alkyl-4-quinolones in Pseudomonas aeruginosa and other bacterial species. Molecular bioSystems 4(9):882-8 doi:10.1039/b803796p

Farrow JM, 3rd, Sund ZM, Ellison ML, Wade DS, Coleman JP, Pesci EC (2008) PqsE functions independently of PqsR-Pseudomonas quinolone signal and enhances the rhl quorum-sensing system. J Bacteriol 190(21):7043-51 doi:10.1128/JB.00753-08

Fujita K, Akino T, Yoshioka H (1988) Characteristics of heat-stable extracellular hemolysin from Pseudomonas aeruginosa. Infect Immun 56(5):1385-7

Gallagher LA, McKnight SL, Kuznetsova MS, Pesci EC, Manoil C (2002) Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa. J Bacteriol 184(23):6472-80

Gunther NWt, Nunez A, Fett W, Solaiman DK (2005) Production of rhamnolipids by Pseudomonas chlororaphis, a nonpathogenic bacterium. Applied and environmental microbiology 71(5):2288-93 doi:10.1128/AEM.71.5.2288-2293.2005

Haimi P, Hermansson M, Batchu KC, Virtanen JA, Somerharju P (2010) Substrate efflux propensity plays a key role in the specificity of secretory A-type phospholipases. J Biol Chem 285(1):751-60 doi:10.1074/jbc.M109.061218

Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, Miller SI (2009) Pseudomonas aeruginosa lasR mutants are associated with cystic fibrosis lung disease progression. J Cyst Fibros 8(1):66-70 doi:10.1016/j.jcf.2008.09.006

Jensen PO, Bjarnsholt T, Phipps R, Rasmussen TB, Calum H, Christoffersen L, Moser C, Williams P, Pressler T, Givskov M, Hoiby N (2007) Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by Pseudomonas aeruginosa. Microbiology 153(Pt 5):1329-38 doi:10.1099/mic.0.2006/003863-0

Koch AK, Kappeli O, Fiechter A, Reiser J (1991) Hydrocarbon assimilation and biosurfactant production in Pseudomonas aeruginosa mutants. J Bacteriol 173(13):4212-9

Kohler T, Guanella R, Carlet J, van Delden C (2010) Quorum sensing-dependent virulence during Pseudomonas aeruginosa colonisation and pneumonia in mechanically ventilated patients. Thorax 65(8):703-10 doi:10.1136/thx.2009.133082

Laabei M, Jamieson WD, Massey RC, Jenkins AT (2014a) Staphylococcus aureus interaction with phospholipid vesicles--a new method to accurately determine accessory gene regulator (agr) activity. PloS one 9(1):e87270 doi:10.1371/journal.pone.0087270

501 Laabei M, Jamieson WD, Massey RC, Jenkins ATA (2014b) Staphylococcus aureus Interaction with
502 Phospholipid Vesicles – A New Method to Accurately Determine Accessory Gene Regulator
503 (agr) Activity. PLoS ONE (In Press)
504 Laabei M, Young A, Jenkins TA (2012) In vitro studies of toxic shock toxin-1-secreting Staphylococcus
505 aureus and implications for burn care in children. Pediatr Infect Dis J 31(5):e73-7
506 doi:10.1097/INF.0b013e3182493b21
507 Leitermann F, Syldatk C, Hausmann R (2008) Fast quantitative determination of microbial
508 rhamnolipids from cultivation broths by ATR-FTIR Spectroscopy. J Biol Eng 2:13
509 doi:10.1186/1754-1611-2-13
510 Marshall SE, Hong SH, Thet NT, Jenkins AT (2013) Effect of lipid and fatty acid composition of
511 phospholipid vesicles on long-term stability and their response to Staphylococcus aureus
512 and Pseudomonas aeruginosa supernatants. Langmuir 29(23):6989-95
513 doi:10.1021/la401679u
514 Mashburn-Warren L, Howe J, Brandenburg K, Whiteley M (2009) Structural requirements of the
515 Pseudomonas quinolone signal for membrane vesicle stimulation. J Bacteriol 191(10):3411-4
516 doi:10.1128/JB.00052-09
517 Mashburn LM, Whiteley M (2005) Membrane vesicles traffic signals and facilitate group activities in
518 a prokaryote. Nature 437(7057):422-5 doi:10.1038/nature03925
519 McKnight SL, Iglewski BH, Pesci EC (2000) The Pseudomonas quinolone signal regulates rhl quorum
520 sensing in Pseudomonas aeruginosa. J Bacteriol 182(10):2702-8
521 Meyer JM, Abdallah MA (1978) The Fluorescent Pigment of Pseudomonas fluorescens : Biosynthesis,
522 Purification and Physicochemical Properties. Journal of General Microbiology 107:319-328
523 Morikawa M, Hirata Y, Imanaka T (2000) A study on the structure-function relationship of
524 lipopeptide biosurfactants. Biochim Biophys Acta 1488(3):211-8
525 Ostroff RM, Vasil AI, Vasil ML (1990) Molecular comparison of a nonhemolytic and a hemolytic
526 phospholipase C from Pseudomonas aeruginosa. J Bacteriol 172(10):5915-23
527 Pamp SJ, Tolker-Nielsen T (2007) Multiple roles of biosurfactants in structural biofilm development
528 by Pseudomonas aeruginosa. J Bacteriol 189(6):2531-9 doi:10.1128/JB.01515-06
529 Pearson JP, Pesci EC, Iglewski BH (1997) Roles of Pseudomonas aeruginosa las and rhl quorum-
530 sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol
531 179(18):5756-67
532 Perfumo A, Rudden M, Smyth TJ, Marchant R, Stevenson PS, Parry NJ, Banat IM (2013) Rhamnolipids
533 are conserved biosurfactants molecules: implications for their biotechnological potential.
534 Applied microbiology and biotechnology 97(16):7297-306 doi:10.1007/s00253-013-4876-z
535 Pesci EC, Milbank JB, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH (1999) Quinolone
536 signaling in the cell-to-cell communication system of Pseudomonas aeruginosa. Proc Natl
537 Acad Sci U S A 96(20):11229-34
538 Pesci EC, Pearson JP, Seed PC, Iglewski BH (1997) Regulation of las and rhl quorum sensing in
539 Pseudomonas aeruginosa. J Bacteriol 179(10):3127-32
540 Pinzon NM, Ju LK (2009) Improved detection of rhamnolipid production using agar plates containing
541 methylene blue and cetyl trimethylammonium bromide. Biotechnol Lett 31(10):1583-8
542 doi:10.1007/s10529-009-0049-7
543 Rikalovic MG, Gojgic-Cvijovic G, Vrvic MM, Karadzic I (2012) Production and characterization of
544 rhamnolipids from Pseudomonas aeruginosa san-ai. Journal of the Serbian Chemical Society
545 77(1):27-42
546 Sanchez M, Aranda FJ, Teruel JA, Espuny MJ, Marques A, Manresa A, Ortiz A (2010) Permeabilization
547 of biological and artificial membranes by a bacterial dirhamnolipid produced by
548 Pseudomonas aeruginosa. J Colloid Interface Sci 341(2):240-7 doi:10.1016/j.jcis.2009.09.042
549 Schenk T, Schuphan I, Schmidt B (1995) High-performance liquid chromatographic determination of
550 the rhamnolipids produced by Pseudomonas aeruginosa. Journal of chromatography A
551 693(1):7-13

552 Schertzer JW, Whiteley M (2012) A bilayer-couple model of bacterial outer membrane vesicle
553 biogenesis. MBio 3(2) doi:10.1128/mBio.00297-11
554 Schuster M, Greenberg EP (2006) A network of networks: quorum-sensing gene regulation in
555 Pseudomonas aeruginosa. Int J Med Microbiol 296(2-3):73-81
556 doi:10.1016/j.ijmm.2006.01.036
557 Schuster M, Sexton DJ, Diggle SP, Greenberg EP (2013) Acyl-homoserine lactone quorum sensing:
558 from evolution to application. Annual review of microbiology 67:43-63 doi:10.1146/annurev-
559 micro-092412-155635
560 Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW,
561 Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV (2006) Genetic adaptation by
562 Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A
563 103(22):8487-92 doi:10.1073/pnas.0602138103
564 Smith RS, Iglewski BH (2003) P. aeruginosa quorum-sensing systems and virulence. Current opinion
565 in microbiology 6(1):56-60
566 Van Delden C, Iglewski BH (1998) Cell-to-cell signaling and Pseudomonas aeruginosa infections.
567 Emerg Infect Dis 4(4):551-60 doi:10.3201/eid0404.980405
568 Wagner VE, Gillis RJ, Iglewski BH (2004) Transcriptome analysis of quorum-sensing regulation and
569 virulence factor expression in Pseudomonas aeruginosa. Vaccine 22 Suppl 1:S15-20
570 doi:10.1016/j.vaccine.2004.08.011
571 Wilder CN, Allada G, Schuster M (2009) Instantaneous within-patient diversity of Pseudomonas
572 aeruginosa quorum-sensing populations from cystic fibrosis lung infections. Infect Immun
573 77(12):5631-9 doi:10.1128/IAI.00755-09
574 Williams P, Camara M (2009) Quorum sensing and environmental adaptation in Pseudomonas
575 aeruginosa: a tale of regulatory networks and multifunctional signal molecules. Current
576 opinion in microbiology 12(2):182-91 doi:10.1016/j.mib.2009.01.005
577 Williams P, Winzer K, Chan WC, Camara M (2007) Look who's talking: communication and quorum
578 sensing in the bacterial world. Philos Trans R Soc Lond B Biol Sci 362(1483):1119-34
579 doi:10.1098/rstb.2007.2039
580 Winzer K, Williams P (2001) Quorum sensing and the regulation of virulence gene expression in
581 pathogenic bacteria. Int J Med Microbiol 291(2):131-43 doi:10.1078/1438-4221-00110
582 Zulianello L, Canard C, Kohler T, Caille D, Lacroix JS, Meda P (2006) Rhamnolipids are virulence
583 factors that promote early infiltration of primary human airway epithelia by Pseudomonas
584 aeruginosa. Infect Immun 74(6):3134-47 doi:10.1128/IAI.01772-05

585